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THERAPEUTIC APPLICATIONS OF FLINT POLYPEPTIDES

BACKGROUND OF THE INVENTION

FasL (also called CD95L and APO1L) is expressed on
5 various cell types and can produce biological responses such
as proliferation, differentiation, immunoregulation,
inflammatory response, cytotoxicity, and apoptosis.
Interestingly, mutations in FasL, the ligand for the TNFR-
family receptor FAS/APO (Suda et al., 1993, Cell 75:1169-78,
10 are associated with autoimmunity (Fisher et al., 1995, Cell
81:935-46; Wu et al. J. Clin. Invest. 5, 1107-1113, 1996),
while overproduction of FasL may be implicated in acute lung
injury (Matute-Bello et al. J. Immun. 163, 2217-2225, 1999).
FasL is expressed in immune-privileged tissues of the eye,
15 testis, brain and some tumors. It has also been found in
kidney and lung as well as in activated thymocytes,
splenocytes, and T lymphocytes.

Apoptosis plays a central role in both development and
in homeostasis. Cells die by apoptosis in the developing
20 embryo during morphogenesis or synaptogenesis and in the
adult animal during tissue turnover or at the end of an
immune response. Because the physiological role of
apoptosis is crucial, aberration of this process can be
detrimental. For example, unscheduled apoptosis of certain
25 brain neurons contributes to disorders such as Alzheimer's
and Parkinson's disease, whereas the failure of dividing
cells to initiate apoptosis after sustaining severe DNA
damage contributes to cancer.

Survival signals from the cell's environment and
30 internal sensors for cellular integrity normally keep a
cell's apoptotic machinery in check. In the event that a
cell loses contact with its surroundings or sustains

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irreparable damage, the cell initiates apoptosis. A cell that simultaneously receives conflicting signals driving or attenuating its division cycle also triggers apoptosis. Mammals have evolved yet another mechanism that enables the organism actively to direct individual cells to self-destruct. This kind of "instructive" apoptosis is important especially in the immune system. Death receptors transmit apoptosis signals initiated by specific "death ligands" and play a central role in instructive apoptosis. These receptors can activate death caspases within seconds of ligand binding, causing an apoptotic demise of the cell within hours.

Death receptors belong to the tumor necrosis factor (TNF) receptor superfamily, which is defined by similar, cysteine-rich extracellular domains. The death receptors contain an additional cytoplasmic sequence termed the "death domain." Death domains typically enable death receptors to engage the cell's apoptotic machinery, but in some instances they mediate functions that are distinct from or even counteract apoptosis.

Fas (also called CD95 or Apo1) is a well-characterized death receptor. Fas and Fas ligand (FasL) play an important role in apoptosis. FasL can form a homotrimeric molecule. It is suggested that each FasL trimer binds to and ligates three Fas molecules. Because death domains have a propensity to associate with one another, Fas ligation leads to clustering of the receptors' death domains. An adapter protein called FADD (Fas-associated death domain; also called Mort1) then binds through its own death domain to the clustered receptor death domains. FADD also contains a "death effector domain" that binds to an analogous domain repeated in tandem within the zymogen form of caspase-8

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(also called FLICE, or MACH). Upon recruitment by FADD, caspase-8 oligomerization drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-9 committing the cell to apoptosis. (Ashkenazi A., et al. "Death Receptors: Signaling and Modulation Science 281, 1305-1308 (August 1998).

Although FasL triggers apoptosis in T lymphocytes, it is proinflammatory. FasL has been shown to stimulate neutrophils, also called polymorphonuclear leukocytes (PMNs), activation. (Chen J. et al., Science 282: 1714-17 (1998)) FasL-Fas binding has been implicated in clonal deletions of autoreactive lymphocytes in peripheral lymphoid tissues, resulting in elimination of autoreactive lymphocyte populations, thus contributing to homeostasis of the immune system. However, it has been found that expression of FasL on myotubes or pancreatic islets of transgenic mice induces a granulocytic response that accelerates graft rejection (Allison J. et al., Proc. Natl. Acad. Sci, 94:3943-47 (April 1997); Kang S-M. et al., Nature Medicien, Vol. 3, No. 7, 738-743 (July 1997)).

At least one of the effects of FasL-Fas receptor binding is apoptosis, which is necessary for homeostasis. However, sometimes the balance of FasL-Fas binding is upset in stress, disease or trauma. One of the negative effects of dysregulated FasL-Fas binding is "runaway" or aberrant apoptosis. Another potential effect of said binding is the destruction of healthy cells caused by neutrophils that have been activated by FasL.

FLINT (i.e. "FAS Ligand Inhibitory Protein) binds FasL and LIGHT, also a member of the TNF family. LIGHT is a membrane-bound ligand that triggers a biological response that is distinct from the Fas-FasL pathway. LIGHT may play a

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role in immune modulation, and in herpes virus entry (see Zhai et al., J. Clin. Invest. 102, 1142-1151, 1998; Montgomery et al. Cell, 87, 427-436, 1996). Soluble LIGHT inhibits the proliferation of various tumor cells whilst
5 binding at least two receptors, LT β R and TR2 (also referred to as herpes virus entry mediator, HVEM). LIGHT is expressed highly in activated lymphocytes and evokes immune modulation from hematopoietic cells. For example, LIGHT stimulates the secretion of IFN γ . LIGHT also induces apoptosis of tumor
10 cells that express the LT β R and TR2/HVEM receptors. The cytotoxic effect of LIGHT is enhanced by IFN γ , which can be blocked by addition of soluble LT β R-Fc or TR2/HVEM-Fc. There is evidence also to support a role for LIGHT in stimulating the proliferation of T cells (See e.g. J. A. Harrop et al.
15 J. Biol. Chem. 273, 27548-556, 1998). Since FLINT binds LIGHT there is thereby a means to inhibit the stimulation of T cells for example as a means to prevent organ rejection following a transplant procedure.

FLINT also interacts with FasL, thereby preventing the
20 binding of FasL to Fas. At least one biological effect results on the binding of FLINT to FasL, namely, inhibition of apoptosis. Since many diseases are thought to involve the FasL-Fas pathway, or the LIGHT-mediated pathway, FLINT could provide a means to treat and/or prevent such diseases.

25 Among the most devastating afflictions to the population of mankind include acute and chronic lung diseases that may involve abnormal apoptosis, for example, lung cancer, pulmonary fibrosis, acute lung injury, connective tissue diseases, drug-induced lung disease,
30 chronic obstructive pulmonary disease and others. There is therefore a need to provide suitable treatments for lung diseases

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SUMMARY OF THE INVENTION

The invention relates to the use of FLINT in treating and/or inhibiting lung disorders, specifically, pulmonary fibrosis ("PF"), and chronic obstructive pulmonary disease ("COPD"), acute lung injury, connective tissue diseases, 5 drug-induced lung disease, and others.

Further aspects of the invention include formulations, having FLINT as an active ingredient that are adapted for treating and/or inhibiting COPD, and PF.

10 Further aspects of the invention include formulations, having FLINT as an active ingredient that are adapted for inhibiting T cell proliferation.

Yet other embodiments of the invention include the use of FLINT in the preparation of medicaments useful in 15 treating and/or inhibiting COPD, and PF.

Yet other embodiments of the invention include the use of FLINT in the preparation of medicaments useful in inhibiting T cell proliferation.

Additionally, the invention includes a method of 20 treating and/or inhibiting COPD in an individual comprising administration of a therapeutically effective amount of FLINT protein to said individual.

Additionally, the invention includes a method of treating and/or inhibiting pulmonary fibrosis in an 25 individual comprising administration of a therapeutically amount of FLINT protein to said individual.

In another embodiment, the present invention relates to the use of FLINT to inhibit T lymphocyte activation.

30 DETAILED DESCRIPTION OF THE INVENTION

SEQ ID NO:1 - Mature human FLINT, i.e. native FLINT minus the leader sequence.

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SEQ ID NO:2 - Nucleic acid/cDNA encoding mature human FLINT.

SEQ ID NO:3 - Native human FLINT.

SEQ ID NO:4 - Human FLINT leader sequence.

5 SEQ ID NO:5 - Nucleic acid/cDNA encoding human FLINT.

Applicants have discovered that FLINT polypeptides are capable of disrupting the FasL-Fas receptor interaction which can cause or exacerbate a variety of diseases.

10 Data presented below show that FLINT inhibits both FasL apoptosis-inducing activity and proinflammatory activity. By antagonizing FasL, FLINT polypeptides can modulate the destruction of healthy cells caused both by neutrophils activated by FasL and by apoptotic damage mediated directly
15 by FasL-Fas interaction. Accordingly, the present methods of treatment utilizing FLINT are useful in the treatment and prevention and/or inhibition of disorders associated with the direct apoptotic effects of FasL and/or the damage mediated by the proinflammatory effects of FasL, whether or
20 not these represent distinct physiological pathways.

Thus, as characterized generally, the invention relates to methods preventing or treating conditions caused or exacerbated by "abnormal apoptosis," in particular, apoptosis induced by Fas ligand (FasL) and Fas receptor
25 (Fas) binding (also referred to as FasL-Fas binding). This invention also relates to methods of preventing or treating conditions caused by a proinflammatory response, more particularly, a proinflammatory response caused by FasL induced neutrophil activation.

30 As used in this application, the term "FLINT" refers to a FLINT polypeptide, for example, a full-length polypeptide including a leader sequence, for example SEQ ID NO:3. The

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term also refers to a FLINT polypeptide lacking a leader, for example SEQ ID NO:1.

As used here, with reference to FasL or Fas expression or interaction, and to any resulting apoptosis, the terms "inappropriate" and "abnormal" should be read to include any deviation from normal expression, interaction or apoptosis levels. Such deviations include temporal, quantitative and qualitative abnormalities. FasL or Fas "expression" refers not only transcription, translation and associated events, but also to any process that results in increased availability of active FasL or Fas, such as transport and/or cell surface availability/accessibility.

Also as used herein the term "abnormal apoptosis" refers to excessive and/or improper apoptosis. Typically abnormal apoptosis is observed in cells and tissues that have undergone physical, chemical or biological insult. Such insults include, but are not limited to physical injury, viral infection, bacterial infection, ischemia, irradiation, chemotherapy, and the like.

The term "effective amount" means an amount of FLINT is capable of treating and/or inhibiting ARDS, ALI, PF and/or COPD and/or in inhibiting T cell activation.

The term "fusion protein" or FLINT fusion protein" denotes a hybrid protein molecule not found in nature comprising a translational fusion or enzymatic fusion in which two or more different proteins or fragments thereof are covalently linked on a single polypeptide chain comprising SEQ ID NO:1 or SEQ ID NO:3, functional fragment thereof.

"Host cell" refers to any eucaryotic or procaryotic cell that is suitable for propagating and/or expressing a cloned gene contained on a vector that is introduced into

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said host cell by, for example, transformation or transfection, or the like.

The term "inhibit" includes the generally accepted meaning, which includes prohibiting, preventing, 5 restraining, slowing, stopping, or reversing progression or severity.

"Isolated nucleic acid compound" refers to any RNA or DNA sequence, however constructed or synthesized, which is locationally distinct from its natural location.

10 "Recombinant DNA cloning vector" as used herein refers to any autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been incorporated.

15 The term "recombinant DNA expression vector" or "expression vector" as used herein refers to any recombinant DNA cloning vector, for example a plasmid or phage, in which a promoter and other regulatory elements are present thereby enabling transcription of an inserted DNA, which may encode 20 a polypeptide.

The term "selectively binding" refers to the ability of FLINT polypeptides to bind FasL but not TNF α .

"Substantially pure" used in reference to a peptide or protein means that said peptide or protein is separated from 25 other cellular and non-cellular molecules, including other protein molecules. A substantially pure preparation would be about at least 85% pure; preferably about at least 95% pure. A "substantially pure" protein as described herein could be prepared by a variety of techniques well known to 30 the skilled artisan, including, for example, the IMAC protein purification method.

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The term "treatment" or "treating" as used herein, describes the management and care of a patient for the purpose of combating a disease, condition, or disorder and includes the administration of FLINT to alleviate the symptoms or complications of said disease, condition, or disorder.

The term "vector" as used herein refers to a nucleic acid compound used for introducing exogenous or endogenous DNA into host cells. A vector comprises a nucleotide sequence which may encode one or more protein molecules. Plasmids, cosmids, viruses, and bacteriophages, in the natural state or which have undergone recombinant engineering, are examples of commonly used vectors.

The various restriction enzymes disclosed and described herein are commercially available and the manner of use of said enzymes including reaction conditions, cofactors, and other requirements for activity are well known to one of ordinary skill in the art. Reaction conditions for particular enzymes were carried out according to the manufacturer's recommendation.

Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of non-accidental death in the United States following heart disease, cancer and cerebral vascular disease. COPD is an obstructive airway disorder encompassing multiple conditions including chronic bronchitis, emphysema, bronchiectasis, and chronic asthma. COPD is slowly progressive and produces an irreversible decline in lung function. Chronic hypoxemia and hypercapnia are the eventual outcomes of the disorder. The mechanism by which COPD disrupts lung function appears to involve dysregulated

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apoptosis. Plasma samples from patients suffering from COPD exhibit higher concentrations of soluble Fas compared with healthy control subjects (See Yasuda et al. Resp. Med. 92, 993-999, 1998). The increased levels of soluble Fas in COPD patients may reflect increased Fas-induced apoptosis.

In another embodiment, the present invention relates to the use of FLINT to treat and/or inhibit COPD in a patient in need thereof by administering a therapeutically effective amount of FLINT.

10 Pulmonary Fibrosis (PF)

Pulmonary fibrosis (also known as fibrosing lung disease) occurs as an end result of the process of attempted healing during acute or chronic lung injury. The pathological mechanism of such lung injury can be any of various factors that first trigger an inflammatory response in the alveoli or surrounding interstitium and subsequently trigger alveolar/interstitial fibrosis (i.e. the repair response). Fibrosis in other tissues such as the epidermis or the peritoneum, leads to visible scarring or adhesions, respectively. Pulmonary fibrosis, in contrast, leads to restrictive lung disease (decreased lung capacities and decreased oxygen diffusion). Conditions associated with pulmonary fibrosis include but are not limited to: idiopathic pulmonary fibrosis, connective tissue diseases (e.g. lupus, scleroderma), drug-induced lung disease (e.g. bleomycin), pneumoconioses (e.g. asbestosis), sarcoidosis, eosinophilic granulomatosis, hypersensitivity pneumonitis, and other diseases associated with severe lung inflammation that can result in acute lung injury and/or acute respiratory distress syndrome (e.g. trauma, sepsis, near-drowning, gastric aspiration, shock, etc.). Fibrosis of the

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airways is also a feature of the chronic inflammation in COPD.

The etiology of PF may involve FasL/Fas-triggered apoptosis. Indeed, an intact FasL/Fas system is essential in the etiology of bleomycin-induced PF in mice (See Kuwano K. et al. J. Clin. Invest. 104, 13-19 (1999)).

In another embodiment the present invention relates to the use of FLINT to inhibit and/or treat PF. For example, FLINT can be administered acutely at the time of an inflammatory insult to the lung (e.g. during bleomycin treatment) to prevent PF from occurring.

Method to inhibit T lymphocyte activation

LIGHT, a member of the tumor necrosis factor superfamily, is a ligand of both the Herpes Simplex Virus Entry Mediator (HVEM) and the lymphotoxin beta receptor (D. N. Mauri et al. Immunity, 8, 21-30, 1998). LIGHT is produced primarily by activated T lymphocytes. When LIGHT binds to HVEM on the surface of T cells it stimulates T cell proliferation (J. A. Harrop et al. J. Biol. Chem. 273, 27548-27556, 1998).

The present invention relates further to the use of FLINT to bind LIGHT, thereby inhibiting T cell activation. T cell activation can be chronically suppressed when advantageous, for example, following organ transplantation to prevent rejection, in the treatment of autoimmune diseases, and in treating systemic inflammatory responses.

Therapeutic Formulations of FLINT

The FLINT polypeptide composition will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the

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individual patient (especially the side effects of treatment with FLINT polypeptide alone), the site of delivery of the FLINT polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners.

An effective amount of polypeptide results in a measurable modulation of the biological activity of the selected TNFR family ligand, for example, FasL or LIGHT. The biological activity for FasL includes, but is not limited to, apoptosis. The biological activity for LIGHT includes, but is not limited to, cell proliferation. LIGHT is a 29 kDa type II transmembrane TNF superfamily member protein produced by activated T cells. (Mauri D.M., Immunity, 8:21-30, January 1998).

Further, an effective amount may also be determined by prevention or amelioration of adverse conditions or symptoms of the diseases or disorders being treated. The "therapeutically effective amount" of FLINT polypeptide for purposes herein is thus determined by such considerations. It should be noted that FLINT is an immunomodulator and that a common observation with such substances is a bell-shaped dose-response curve. Such a phenomenon is well known in the art and it is within the skill of the clinician to take this into account in adjusting the therapeutically effective amount of FLINT accordingly.

As a general proposition, the total pharmaceutically effective amount of FLINT polypeptide administered parenterally per dose will be in the range of about 1 µg/kg/day to 10 mg/kg/day of patient body weight, more particularly 2-8mg/kg, preferably 2-4mg/kg, most preferred 2.2mg/kg to 3.3 mg/kg and finally 2.5 mg/kg. However, as

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noted above, this will be subject to therapeutic discretion. Preferably, this dose is at least 0.01 mg/kg/day.

If given continuously, the FLINT polypeptide is typically administered at a dose rate of about 0.1

5 $\mu\text{g/kg/hour}$ to about 50 $\mu\text{g/kg/hour}$, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses
10 to occur appears to vary depending on the desired effect.

Pharmaceutical compositions containing the FLINT of the invention may be administered using a variety of modes that include, but are not limited to, oral, rectal, intra-
cranial, parenteral, intracisternal, intrathecal,
15 intravaginal, intraperitoneal, intratracheal, intrabronchopulmonary, topical, transdermal (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid
20 filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include but are not limited to, intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and
25 infusion. Implants comprising FLINT also can be used.

The FLINT polypeptide is also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or
30 microcapsules. Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate

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(Sidman, U. et al., *Biopolymers* 22:547-556 (1983)), poly (2-hydroxyethyl methacrylate) (R. Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981), and R. Langer, *Chem. Tech.* 12:98-105 (1982)), ethylene vinyl acetate (R. Langer et al.,
5 Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988). Sustained-release FLINT polypeptide compositions also include liposomally entrapped FLINT polypeptides. Liposomes containing FLINT polypeptides are prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci.*
10 (USA) 82:3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. (USA)* 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EDP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about
15 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal TNFR polypeptide therapy.

For parenteral administration, in one embodiment, the
20 FLINT polypeptide is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations
25 employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting
30 the FLINT polypeptide uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired

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formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and
5 dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and
10 chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight
15 (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides,
20 disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.
25 The FLINT polypeptide is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the
30 formation of FLINT polypeptide salts.

FLINT polypeptides to be used for therapeutic administration must be sterile. Sterility is readily

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accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic FLINT polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

FLINT polypeptides ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous FLINT polypeptide solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized FLINT polypeptide using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

Polypeptide Production Methods

The FLINT polypeptides and fusion polypeptides of this invention may be synthesized recombinantly as disclosed in U.S. Patent Application Serial Number 09/280,567 herein incorporated by reference. It often is observed in the

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production of certain peptides in recombinant systems that expression as a fusion polypeptide prolongs the life span, increases the yield of the desired peptide, or provides a convenient means of purifying the polypeptide. This is particularly relevant when expressing mammalian polypeptides in procaryotic hosts. A variety of peptidases (e.g. enterokinase and thrombin) which cleave a polypeptide at specific sites or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase) of the peptide chain are known. Furthermore, particular chemicals (e.g. cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. For instance, see P. Carter, "Site Specific Proteolysis of Fusion Polypeptides", Chapter 13, in PROTEIN PURIFICATION: FROM MOLECULAR MECHANISMS TO LARGE SCALE PROCESSES, American Chemical Society, Washington, D.C. (1990).

In addition to prokaryotes, a variety of amphibian expression systems such as frog oocytes, and mammalian cell systems can be used. The choice of a particular host cell depends to some extent on the particular expression vector used. Exemplary mammalian host cells suitable for use in the present invention include HepG-2 (ATCC HB 8065), CV-1 (ATCC CCL 70), LC-MK₂ (ATCC CCL 7.1), 3T3 (ATCC CCL 92), CHO-K1 (ATCC CCL 61), HeLa (ATCC CCL 2), RPMI8226 (ATCC CCL 155), H4IIEC3 (ATCC CCL 1600), C127I (ATCC CCL 1616), HS-Sultan (ATCC CCL 1484), and BHK-21 (ATCC CCL 10), for example.

A wide variety of vectors are suitable for transforming mammalian host cells. For example, the pSV2-type vectors

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comprise segments of the simian virus 40 (SV40) genome required for transcription and polyadenylation. A large number of plasmid pSV2-type vectors have been constructed, such as pSV2-gpt, pSV2-neo, pSV2-dhfr, pSV2-hyg, and pSV2--
5 globin, in which the SV40 promoter drives transcription of an inserted gene. These vectors are widely available from sources such as the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, or the National Center for Agricultural Utilization Research, 1815
10 North University Street, Peoria, Illinois 61604-39999.

Promoters suitable for expression in mammalian cells include the SV40 late promoter, promoters from eucaryotic genes, such as, for example, the estrogen-inducible chicken ovalbumin gene, the interferon genes, the glucocorticoid-
15 inducible tyrosine aminotransferase gene, the thymidine kinase gene promoter, and the promoters of the major early and late adenovirus genes.

Plasmid pRSVcat (ATCC 37152) comprises portions of a long terminal repeat of the Rous Sarcoma virus, a virus known to
20 infect chickens and other host cells. This long terminal repeat contains a promoter which is suitable for use in the vectors of this invention. H. Gorman et al., *Proc. Nat. Acad. Sci. (USA)*, 79, 6777 (1982). The plasmid pMSVi (NRRL B-15929) comprises the long terminal repeats of the Murine
25 Sarcoma virus, a virus known to infect mouse and other host cells. The mouse metallothionein promoter has also been well characterized for use in eucaryotic host cells and is suitable for use in the present invention. This promoter is present in the plasmid pdBPV-MMTneo (ATCC 37224) which
30 can serve as the starting material for the construction of other plasmids of the present invention.

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Transfection of mammalian cells with vectors can be performed by a plurality of well known processes including, but not limited to, protoplast fusion, calcium phosphate co-precipitation, electroporation and the like. See, e.g.,

5 Maniatis et al., *supra*.

Some viruses also make appropriate vectors. Examples include the adenoviruses, the adeno-associated viruses, the vaccinia virus, the herpes viruses, the baculoviruses, and the Rous sarcoma virus, as described in U.S. Patent

10 4,775,624, incorporated herein by reference. For example, the baculovirus pFastBac-1 (GIBCO/BRL) can be used to infect a suitable host cell, such as SF9, to produce recombinant protein.

Eucaryotic microorganisms such as yeast and other fungi

15 are also suitable host cells. The yeast *Saccharomyces cerevisiae* is the preferred eucaryotic microorganism. Other yeasts such as *Kluyveromyces lactis* and *Pichia pastoris* are also suitable. For expression in *Saccharomyces*, the plasmid YRp7 (ATCC-40053), for example, may be used. See, e.g., L.

20 Stinchcomb et al., *Nature*, 282, 39 (1979); J. Kingsman et al., *Gene*, 7, 141 (1979); S. Tschemper et al., *Gene*, 10, 157 (1980). Plasmid YRp7 contains the TRP1 gene which provides a selectable marker for use in a *trp1* auxotrophic mutant.

The following examples more fully describe the present

25 invention. Those skilled in the art will recognize that the particular reagents, equipment, and procedures described are merely illustrative and are not intended to limit the present invention in any manner.

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EXAMPLE 1RT-PCR Amplification of FLINT Gene from mRNA

A FLINT gene is isolated by reverse transcriptase PCR (RT-PCR) using conventional methods. Total RNA from a tissue that expresses the FLINT gene, for example, lung, is prepared using standard methods. First strand FLINT cDNA synthesis is achieved using a commercially available kit (SuperScript™ System; Life Technologies) by PCR in conjunction with specific primers directed at any suitable region of the FLINT gene (e.g. SEQ ID NO:2 or SEQ ID NO:5). Amplification is carried out by adding to the first strand cDNA (dried under vacuum): 8 µl of 10X synthesis buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl, 25 mM MgCl₂, 1 ug/ul BSA); 68 µl distilled water; 1 µl each of a 10 uM solution of each primer; and 1 µl Taq DNA polymerase (2 to 5 U/µl). The reaction is heated at 94° C for 5 minutes to denature the RNA/cDNA hybrid. Then, 15 to 30 cycles of PCR amplification are performed using any suitable thermal cycle apparatus. The amplified sample may be analyzed by agarose gel electrophoresis to check for an appropriately-sized fragment.

EXAMPLE 2Production of a Vector for Expressing FLINT in a Host Cell

An expression vector suitable for expressing FLINT or fragment thereof in a variety of procaryotic host cells, such as *E. coli* is easily made. The vector contains an origin of replication (Ori), an ampicillin resistance gene (Amp) useful for selecting cells which have incorporated the vector following a transformation procedure, and further comprises the T7 promoter and T7 terminator sequences in operable linkage to a FLINT coding region. Plasmid pET11A

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(obtained from Novogen, Madison WI) is a suitable parent plasmid. pET11A is linearized by restriction with endonucleases NdeI and BamHI. Linearized pET11A is ligated to a DNA fragment bearing NdeI and BamHI sticky ends and comprising the coding region of the FLINT gene as disclosed by SEQ ID NO:5.

The FLINT gene used in this construction may be slightly modified at the 5' end (amino terminus of encoded polypeptide) in order to simplify purification of the encoded polypeptide product. For this purpose, an oligonucleotide encoding 8 histidine residues is inserted after the ATG start codon. Placement of the histidine residues at the amino terminus of the encoded polypeptide serves to enable the IMAC one-step polypeptide purification procedure.

EXAMPLE 3

Recombinant Expression and Purification of FLINT Polypeptide

An expression vector that carries an open reading frame (ORF) encoding FLINT or fragment thereof and which ORF is operably-linked to an expression promoter is transformed into *E. coli* BL21 (DE3) (*hsdS gal •cIts857 ind1Sam7nin5lacUV5-T7gene 1*) using standard methods. Transformants, selected for resistance to ampicillin, are chosen at random and tested for the presence of the vector by agarose gel electrophoresis using quick plasmid preparations. Colonies which contain the vector are grown in L broth and the polypeptide product encoded by the vector-borne ORF is purified by immobilized metal ion affinity chromatography (IMAC), essentially as described in US Patent 4,569,794.

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Briefly, the IMAC column is prepared as follows. A metal-free chelating resin (e.g., Sepharose 6B IDA, Pharmacia) is washed in distilled water to remove preservative substances and infused with a suitable metal ion [e.g., Ni(II), Co(II), or Cu(II)] by adding a 50mM metal chloride or metal sulfate aqueous solution until about 75% of the interstitial spaces of the resin are saturated with colored metal ion. The column is then ready to receive a crude cellular extract containing the recombinant polypeptide product.

After removing unbound polypeptides and other materials by washing the column with any suitable buffer, pH 7.5, the bound polypeptide is eluted in any suitable buffer at pH 4.3, or preferably with an imidazole-containing buffer at pH 7.5.

EXAMPLE 4

Construction of Mammalian FLINT-non-Flag Expression Vector

In order to generate a non-Flagged expression vector (pIG1-FLINT), the 24-base DNA sequence encoding the eight amino acid FLAG epitope was deleted from the pIG1-FLINTF construct using the Quick Change mutagenesis kit (Stratagene). A 35-base primer, and its complement, with identity to the 19-base sequences flanking the FLAG sequence was synthesized and used for PCR amplification using pIG1-FLINTF the plasmid as template. The PCR reaction mixture was digested with *DpnI* restriction endonuclease to eliminate the parental DNA, and the PCR product was transformed into Epicurean XLI-blue *E. coli* cells. Sixteen ampicillin-resistant transformants were picked and the plasmid DNA was analyzed by restriction digestion. Ten of the 16 gave results compatible with deletion of the 24-base sequence.

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Precise deletion of the 24-base sequence was confirmed by DNA sequencing of pIG1-FLINT.

EXAMPLE 5

5 FAS LIGAND BINDING EXPERIMENTS

Dot blot experiment was performed to scan known TNF ligands that are commercially available TRAIL and FasL for interaction with FLINT.

TRAIL (RnD Systems) and FasL (Kamiya Biomedical
10 Company) were spotted on a nitrocellulose paper and incubated with purified FLINT-Flag. FLINT was washed away and binding FLINT was detected using anti Flag antibody. Both OPG2Fc and FLINT-Flag were overexpressed and purified according the examples above. The filter paper was
15 subsequently blocked for 30 min using 5% nonfat milk in PBS in room temperature. The nitrocellulose paper was subsequently mixed with the cell lysate containing FasL-Myc, and further incubated on a rotator for 1 hour at room temperature. Secondary and tertiary incubations were
20 performed with anti-myc antibody and anti-mouse IgG-HRP for 1 hour and 30 minutes respectively. The polypeptide containing myc epitope was detected by chemiluminescence on X-ray film which showed that FLINT bound to FasL specifically. No appreciable binding was detected with
25 TNF•, TNF•, TRAIL, CD40L or TRANCE.

First a baseline experiment was done for the Fas-FasLigand interaction *in vitro*. Unless otherwise indicated, all washing steps use TBST (Tris Buffer Saline with Tween 20 from SIGMA) and were done 3 to 6 times.

30 Fas (100 ng) was adsorbed on to ELISA plate. Then the plate was is blocked by TBST plus 0.1% Gelatine. Thereafter, FasL (Flag-tagged) was added at different

-24-

concentrations with a maximum concentration of 300 ng going down to 1 ng on TBST plus a 0.1% solution containing 1 micrograms/ml of M2 Abs (antiflag antibodies purchased through Scientific Imaging System division of Kodak). After washing the plate 6 times, anti-mouse-Abs-HRP (3000 dilution, Bio-Rad) was added to the wells. After washings three times, visualization enzymatic reaction using ABTS as a substrate was performed. Unless otherwise noted, an ELISA reader commercialized by Molecular Devices Corp. (Menlo Park, California) was used.

The following data were collected:

FasL, ng	OD, 405nm
1	.1
5	.2
10	.3
50	.7
100	1.2
500	1.6

FLINT-Fas L binding may be confirmed and specifics of binding determined (e.g., kinetics, specificity, affinity, cooperativity, relative binding pattern, concentration) using real-time biomolecular interaction analysis. This technology confers the ability to study biomolecular interactions in real time, without labeling any of the interactants. In particular, it takes advantage of the optical phenomenon surface plasmon resonance, and detection depends on changes in the mass concentration of macromolecules at the biospecific interface. Interactions are followed in real time, so that kinetic information is readily derived. In many cases, investigations can be performed without prior purification of components.

Measurements are accomplished using a BiaCore 2000 instrument. The instrument, accompanying chips,

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immobilization and maintenance kits and buffers are obtained from Biacore AB, Rapskatan 7, S-754 50 Uppsala, Sweden. FasL is obtained from Kamiya Biomedical Company, 910 Industry Drive, Seattle, WA 98188, Guanidine Isothiocyanate Solution from GibcoBRL.

EXAMPLE 6

Measuring the effect of FLINT on anti-CD3 induced Jurkat apoptosis

10 Non-tissue treated 24 well plates (Decton Dickinson, Mansfield, MA) were coated with 0.5 ml of 1 ug/ml anti-CD3 (Farmingden) in PBS for 90 min at 37 °C. The plate was washed once with PBS. 1 ml of 1×10^6 cell/ml was seated in each well with or without following treatment: 10 μ M DEVD-cmk, 1 ug OPG2-Fc, 1 or 2 ug of FLINT and 1 ug anti FasL Ab. 15 FLINT was made according to Examples 3.

Cells were incubated overnight at 37 °C incubator and cells were then stained by Annexin V and PI staining. Apoptosis was analyzed by flow cytometer (FACS). Cell 20 apoptosis was indicated by positive staining with Annexin V.

Control Jurkat	6.97
Jurkat + anti Fas	59.28
Jurkat + antiCD3	46.32
Jurkat + antiCD3 + DEVDcmk	30.80
Jurkat + antiCD3 + FLINT (1ug)	27.77
Jurkat + antiCD3 + OPG2-Fc (1ug)	45.78
Jurkat + antiCD3 + FLINT (2ug)	18.67
Jurkat + antiCD3 + antiFasL Ab	24.05

EXAMPLE 7

Measuring the effect of FLINT on recombinant FasL induced Jurkat cells apoptosis

25 One milliliter of 1×10^6 cell/ml was added into each well of 24 well tissue culture plate and treated with

-26-

following reagents: soluble Fas L (200 ng), Fas L plus 1 ug FLINT, Fas L plus 1 ug OPG2-Fc, Trail (200 ng), Trail plus 1 ug FLINT. Cells were incubated overnight at 37 °C and then stained with Annexin V and PI. Cell apoptosis was analyzed
 5 by flow cytometer (FACS). FLINT was made according to Examples 3.

Control Jurkat	3.23
Jurkat + FasL (200ng/ml)	67.39
Jurkat + FasL (200ng/ml) + anti FasL Ab (1 ug)	3.3
Jurkat + FasL (200ng/ml) + FLINT (1 ug)	3.32
Jurkat + FasL (200ng/ml) + FLINT (1 ug)	4.6
Jurkat + FasL (200ng/ml) + OPG2 (1ug)	70.58
Jurkat + FasL (200ng/ml) + OPG2 (1ug)	69.58
Jurkat + TRAIL (200ng/ml)	17.47
Jurkat + TRAIL (200ng/ml)	17.43

EXAMPLE 8

10 Measuring the effect of FLINT in a dose-dependent
 manner on anti-CD3 induced Jurkat apoptosis

The same steps for plate coating and cell treatment set out in Example 7 were followed except a different amount of FLINT was added into each well. FLINT was made according to
 15 Examples 3. The following table indicates the amounts added:

-27-

Jurkat cells (Control)	5.33
Jurkat cells + anti CD3	27.49
Jurkat cells + anti CD3 + anti FasL neutralization Ab	12.74
Jurkat cells + anti CD3 + OPG2-Fc 4ug	26.24
Jurkat cells + anti CD3 + FLINT/PG3 3000ng	14.68
Jurkat cells + anti CD3 + FLINT 2000ng	17.02
Jurkat cells + anti CD3 + FLINT 1000ng	24.29
Jurkat cells + anti CD3 + FLINT 500ng	27.48
Jurkat cells + anti CD3 + FLINT 250ng	28.93
Jurkat cells + anti CD3 + FLINT 125ng	29.4
Jurkat cells + anti CD3 + FLINT 62.5ng	28.99
Jurkat cells + anti CD3 + FLINT 31.25ng	28.21
Jurkat cells + anti CD3 + FLINT 15.625ng	28.80

EXAMPLE 9

Use of FLINT to Treat ALI Patient

5 A 55 year-old male presents to the emergency department unconscious. His family states that he was being treated as an outpatient for bronchitis for the past few days but worsened despite antibiotics. He has no relevant past history and his only medication was a third generation oral

10 cephalosporin. Physical examination reveals an obtunded, cyanotic male who is hypotensive, tachypneic, and tachycardic, and who has bilateral lung congestion consistent with pulmonary edema. There is no evidence of congestive heart failure. Tests reveal hypoxemia (based on

15 PaO₂/FiO₂) and bilateral lung infiltrates without cardiomegaly, consistent with a diagnosis of acute lung

-28-

injury. Based on the history it is concluded that the lung injury was a direct result of community-acquired pneumonia, and that the patient met the hypoxemia criteria for ALI within the last 12 hours. Ventilation measures include use
5 of PEEP and low tidal volume. As soon as adequate oxygenation is confirmed, treatment with FLINT is initiated in the ER as an iv bolus of 2.5 mg/kg, followed by a continuous infusion of 0.1 mg/minute. FLINT along with aggressive supportive measures (e.g., positive ventilation,
10 intravenous fluids, pressors, and nutritional support) are continued for four days in the ICU, at which time the FLINT is discontinued. Over the following 3 days, the patient begins to recover and is extubated on Day 8. He has an uneventful recovery and 6 months following discharge has no
15 evidence of residual lung disease by blood gas and spirometry.

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We claim:

1. FLINT for use in the treatment and/or inhibition of a lung disease.
- 5 2. FLINT, as in claim 1, for use in treating and/or inhibiting pulmonary fibrosis.
3. FLINT, as in claim 1, for use in treating and/or
10 inhibiting chronic obstructive pulmonary disease.
4. Use of FLINT in the manufacture of a medicament for the treatment and/or inhibition of a lung disease.
- 15 5. Use of FLINT, as in claim 4, in the manufacture a medicament for the treatment and/or inhibition of pulmonary fibrosis.
6. Use of FLINT to manufacture a medicament for inhibiting
20 T cell activation.
7. Use of FLINT, as in claim 4, in the manufacture of a medicament for the treatment and/or inhibition of chronic obstructive pulmonary disease.
- 25 8. A method for treating pulmonary fibrosis comprising administering to a patient in need thereof an effective amount of FLINT.
- 30 9. A method for inhibiting pulmonary fibrosis comprising administering to a patient in need thereof an effective amount of FLINT.

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10. A method for treating chronic obstructive pulmonary disease comprising administering to a patient in need thereof an effective amount of FLINT.
- 5 11. A method for inhibiting chronic obstructive pulmonary disease comprising administering to a patient in need thereof an effective amount of FLINT.
- 10 12. A method for inhibiting T cell activation comprising administering to a patient in need thereof an effective amount of FLINT.

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- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: THERAPEUTIC APPLICATIONS OF FLINT POLYPEPTIDES

(57) Abstract: FLINT protein is useful as a medicament in treating and/or inhibiting lung disorders and in inhibiting T cell proliferation. Therapeutic compositions and methods of treatment utilizing FLINT are provided.



WO 01/28582 A3

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PTO/SB/01 (8-96) (MODIFIED)
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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

☒ Declaration Submitted with Initial Filing
☐ Declaration Submitted after Initial Filing

Attorney Docket Number	X-13199
First Named Inventor	Thomas Frank Bumol, et al.
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

THERAPEUTIC APPLICATIONS OF FLINT POLYPEPTIDES

the specification of which

☐ is attached hereto

OR

☒ was filed on
(MM/DD/YYYY)

10/06/2000

as United States Application Number or PCT International

Application
Number

PCT/US00/26241

and was amended on
(MM/DD/YYYY)

(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached	
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☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional applications(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/160,613	10/20/1999

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America; listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

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Mark J. Stewart	43,936
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Robert C. Tucker	45,166
Tina M. Tucker	47,145
MaCharri Vorndran-Jones	36,711
Gilbert T. Voy	43,872
Thomas D. Webster	39,872
Lawrence T. Welch	29,487
Alexander Wilson	45,782
Dan L. Wood	48,613

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

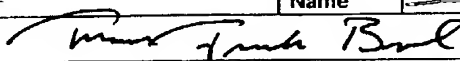
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Country	US	Telephone	(317) 276-3334
		Fax	(317) 276-3861

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:

☐ A Petition has been filed for this unsigned inventor

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☒ Additional Inventors are being named on supplement sheet(s) attached hereto.

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION

Name of Additional Joint Inventor, if any:				<input type="checkbox"/> A Petition has been filed for this unsigned inventor			
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Inventor's Signature	<i>Fred Jay Cohen</i>				Date	01-Apr-2002	
Residence: City	Newtown	State	PA	Country	US	Citizenship	US
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tcg ctg ctg tgc ctg gtg ttg gcg ctg cct gcc ctg ctg ccg gtg ccg      99
Ser Leu Leu Cys Leu Val Leu Ala Leu Pro Ala Leu Leu Pro Val Pro
      10                      15                      20                      25

gct gta cgc gga gtg gca gaa aca ccc acc tac ccc tgg cgg gac gca      147
Ala Val Arg Gly Val Ala Glu Thr Pro Thr Tyr Pro Trp Arg Asp Ala
              30                      35                      40

gag aca ggg gag cgg ctg gtg tgc gcc cag tgc ccc cca ggc acc ttt      195
Glu Thr Gly Glu Arg Leu Val Cys Ala Gln Cys Pro Pro Gly Thr Phe
              45                      50                      55

gtg cag cgg ccg tgc cgc cga gac agc ccc acg acg tgt ggc ccg tgt      243
Val Gln Arg Pro Cys Arg Arg Asp Ser Pro Thr Thr Cys Gly Pro Cys
              60                      65                      70

cca ccg cgc cac tac acg cag ttc tgg aac tac ctg gag cgc tgc cgc      291
Pro Pro Arg His Tyr Thr Gln Phe Trp Asn Tyr Leu Glu Arg Cys Arg
              75                      80                      85

tac tgc aac gtc ctc tgc ggg gag cgt gag gag gag gca cgg gct tgc      339
Tyr Cys Asn Val Leu Cys Gly Glu Arg Glu Glu Glu Ala Arg Ala Cys
      90                      95                      100                      105

cac gcc acc cac aac cgt gcc tgc cgc tgc cgc acc ggc ttc ttc gcg      387
His Ala Thr His Asn Arg Ala Cys Arg Cys Arg Thr Gly Phe Phe Ala
              110                      115                      120

cac gct ggt ttc tgc ttg gag cac gca tcg tgt cca cct ggt gcc ggc      435
His Ala Gly Phe Cys Leu Glu His Ala Ser Cys Pro Pro Gly Ala Gly
              125                      130                      135

gtg att gcc ccg ggc acc ccc agc cag aac acg cag tgc cag ccg tgc      483
Val Ile Ala Pro Gly Thr Pro Ser Gln Asn Thr Gln Cys Gln Pro Cys
              140                      145                      150

ccc cca ggc acc ttc tca gcc agc agc tcc agc tca gag cag tgc cag      531
Pro Pro Gly Thr Phe Ser Ala Ser Ser Ser Ser Ser Glu Gln Cys Gln
      155                      160                      165

ccc cac cgc aac tgc acg gcc ctg ggc ctg gcc ctc att gtg cca ggc      579
Pro His Arg Asn Cys Thr Ala Leu Gly Leu Ala Leu Ile Val Pro Gly
      170                      175                      180                      185

tct tcc tcc cat gac acc ctg tgc acc agc tgc act ggc ttc ccc ctc      627
Ser Ser Ser His Asp Thr Leu Cys Thr Ser Cys Thr Gly Phe Pro Leu
              190                      195                      200

agc acc agg gta cca gga gct gag gag tgt gag cgt gcc gtc atc gac      675
Ser Thr Arg Val Pro Gly Ala Glu Glu Cys Glu Arg Ala Val Ile Asp

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205										210					215					
ttt	gtg	gct	ttc	cag	gac	atc	tcc	atc	aag	agg	ctg	cag	cgg	ctg	ctg	723				
Phe	Val	Ala	Phe	Gln	Asp	Ile	Ser	Ile	Lys	Arg	Leu	Gln	Arg	Leu	Leu					
		220						225					230							
cag	gcc	ctc	gag	gcc	ccg	gag	ggc	tgg	gct	ccg	aca	cca	agg	gcg	ggc	771				
Gln	Ala	Leu	Glu	Ala	Pro	Glu	Gly	Trp	Ala	Pro	Thr	Pro	Arg	Ala	Gly					
	235					240					245									
cgc	gcg	gcc	ttg	cag	ctg	aag	ctg	cgt	cgg	cgg	ctc	acg	gag	ctc	ctg	819				
Arg	Ala	Ala	Leu	Gln	Leu	Lys	Leu	Arg	Arg	Arg	Leu	Thr	Glu	Leu	Leu					
250					255				260						265					
ggg	gcg	cag	gac	ggg	gcg	ctg	ctg	gtg	cgg	ctg	ctg	cag	gcg	ctg	cgc	867				
Gly	Ala	Gln	Asp	Gly	Ala	Leu	Leu	Val	Arg	Leu	Leu	Gln	Ala	Leu	Arg					
				270					275					280						
gtg	gcc	agg	atg	ccc	ggg	ctg	gag	cgg	agc	gtc	cgt	gag	cgc	ttc	ctc	915				
Val	Ala	Arg	Met	Pro	Gly	Leu	Glu	Arg	Ser	Val	Arg	Glu	Arg	Phe	Leu					
			285					290					295							
cct	gtg	cac	tgatcctggc	cc												936				
Pro	Val	His																		
		300																		